

**PCT**WORLD INTELLECTUAL PRO  
International I

INTERNATIONAL APPLICATION PUBLISHED UNDER

(51) International Patent Classification<sup>6</sup>:**C12N 15/82, 15/60, 5/10, A01H 5/00****A1**

(11) I

**WO 9607746A1**

(43) International Publication Date:

**14 March 1996 (14.03.96)**(21) International Application Number: **PCT/US95/11199**(22) International Filing Date: **5 September 1995 (05.09.95)**

(30) Priority Data:

**08,303,266****8 September 1994 (08.09.94)****US**

(60) Parent Application or Grant

(63) Related by Continuation

**US****08/303,266 (CIP)**

Filed on

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## (57) Abstract

The present invention is directed to isolated non-coding nucleotide sequences useful as promoters for heterologous gene expression in plants. The present invention is also directed to vectors and plant cells comprising the isolated nucleotide sequences. AHAS promoters from maize are used to express introduced genes at high levels and in various plant tissues. Promoters from the *als1* and *als2* genes of maize are cloned and sequenced, and the promoter regions from these genes are then introduced into a plasmid 5' to the reporter gene beta-glucuronidase (GUS). Both promoter fragments are from the XI12 maize line. The *als1* promoter fragment is approximately 1400 base pairs long, whereas the *als2* promoter fragment contains 819 base pairs.

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Acetohydroxyacid synthase promoter for expression of  
introduced genes in plants

FIELD OF THE INVENTION

The present invention is directed to isolated non-coding nucleotide sequences useful as promoters for heterologous gene expression in plants. The present invention is also directed to vectors and plant cells comprising the isolated nucleotide sequences.

BACKGROUND OF THE INVENTION

The Plant Kingdom is divided into two phyla, the Bryophyta and Tracheophyta. The phylum Tracheophyta includes over 266,000 species grouped into four subphyla. Subphylum pteropsida includes Class Angiospermae. This class is divided into two subclasses, the dicotyledons (dicots) and the monocotyledons (monocots).

Since monocots include many of the important grain and feed crops, plant geneticists are keenly interested in being able to produce transgenic monocots. About 50,000 species of monocots are known. These include the lilies, palms, orchids, irises, tulips, sedges, and grasses. The grasses include corn, wheat, rice and all other cereal grains. Unfortunately, monocots have been extremely difficult to genetically engineer so that most of the work with plants has been with dicots.

The dicots are the larger of the two groups, with approximately 200,000 species known. The buttercup, snapdragon, carnation, magnolia, poppy, cabbage, rose, pea, poinsettia, cotton plant, cactus, carrot, blueberry, mint, tomato, sunflower, elm, oak,

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and maple represent 19 of the 250 families of dicots.

Th g netic information within a DNA molecule usually serves as the template for the synthesis of a large number of shorter RNA molecules, most of which in turn serve as templates for the synthesis of specific polypeptide chains. Specific nucleotide segments, (often called promoters), are recognized by RNA polymerase molecules that start RNA synthesis. After transcription of a functional RNA chain is finished, a second class of signals leads to the termination of RNA synthesis and the detachment of RNA polymerase molecules from their respective DNA templates.

Currently, there are a number of common promoters used to drive heterologous gene expression in monocot plants.

David McElroy, et al., (1990) noted that transient expression assays of a construct in which the promoter from the rice actin 1 gene (Act 1) was fused to the bacterial  $\beta$ -glucuronidase gene (GUS) in transformed rice protoplasts showed that the actin promoter drives high levels of gene expression. This expression was about 6-fold greater than that seen with the maize alcohol dehydrogenase 1 gene (Adh1) promoter, and is dependent on the presence of an intact Act1 5' intron.

David McElroy, et al., (1991) noted that optimized vectors for monocot transformation were constructed using either the Cauliflower Mosaic Virus (CaMV) 35S promoter or the Act1 promoter. Transient expression assays were done on both transformed rice and maize protoplasts. Addition of the Act1 intron and optimized GUS translation initiation site to either promoter sequence increased gene expression significantly. It is also noted as an unpublished result that the actin promoter was shown to driv GUS

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expression in transient assays of wheat, oat, barley and sorghum protoplasts.

5 Wanggen Zhang, et al., (1991) noted that *in situ* hybridization studies of transgenic rice plants carrying an Act1-GUS gene fusion showed that the Act1 promoter has a constitutive pattern of expression in both vegetative and reproductive tissue.

10 Jun Cao, et al., (1992) noted that transgenic rice plants were selected on bialophos following transformation with the *bar* gene expressed under the control of either the CaMV 35S promoter or the rice Act1 promoter.

15 Alan H. Christensen, et al., (1992) noted that Maize protoplasts transformed with a maize Ubi-1-CAT gene fusion showed approximately 10-fold higher levels of CAT activity than maize cells transformed with a CaMV-35S-GUS gene fusion in transient expression assays. Northern blot analysis of Ubi-1 and Ubi-2 transcript levels following heat shock of  
20 maize seedlings demonstrated that both genes are expressed constitutively at 25C, but are induced following the heat shock.

25 Seiichi Toki, et al., (1992) noted that stably transformed transgenic rice plants were obtained following electroporation-mediated transformation of the *bar* gene expressed under the control of the maize Ubi-1 promoter and selection on bialophos. This result demonstrates that the Ubi-1 promoter can be used to drive sufficiently high levels  
30 of gene expression in rice to allow for selection and regeneration of fertile, transgenic rice plants.

35 J. Troy, et al., (1993) noted that stably transformed wheat plants were obtained following bombardment of calli derived from immature embryos with both the *bar* gene and GUS, each being expressed under the control of the maize Ubi-1 promoter,

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followed by selection on bialaphos. This result demonstrates that the Ubi-1 promoter can be used to drive sufficiently high levels of gene expression in wheat to allow for selection and regeneration of fertile, transgenic plants.

Yuechun Wan, and Peggy G. Lemaux, (1994) noted that stably transformed, fertile barley plants were obtained after microprojectile bombardment of embryonic barley tissues with both the bar gene and GUS, each being expressed under the control of the maize Ubi-1 promoter, followed by selection on bialaphos. This result demonstrates that the Ubi-1 promoter can be used to drive sufficiently high levels of gene expression in barley to allow for selection and regeneration of fertile, transgenic plants. An experiment involving bombardment of a small number of plants with either Ubi-bar or CaMV 35S-bar showed no significant difference in the number of transformants obtained.

Junko Kyoizuka, et al., (1991) noted that a maize Adh1 promoter-GUS fusion was introduced into rice protoplasts to obtain transgenic rice plants. The GUS activity in the transgenic plants was examined to determine the pattern of GUS expression. The maize Adh1 promoter was found to promote constitutive expression in all parts of the plants examined. As had been previously demonstrated for Adh1 expression in maize, the Adh1-driven GUS expression was induced in roots by anaerobic conditions.

D. I. Last, et al., (1991) noted that when the maize Adh1 promoter was modified by addition of multiple copies of the Anaerobic Responsive element of the maize ADH1 gene and ocs elements from the octopine synthase gene of *Agrobacterium tumefaciens* (pEmu). In transient expression assays of protoplasts of different monocot species transformed with pEmu-GUS,

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the best construct, gave 10-50-fold higher levels of expression than the CaMV 35S promoter in wheat, maize, rice, einkorn, and lolium multiflorum.

5 Robert Bower and Robert G. Birch (1992) noted that stable transformants were obtained following transformation of embryogenic callus of sugarcane with the neomycin phosphotransferase gene under the control of the Emu promoter.

10 D.A. Chamberlain, et al., (1994) noted that the Emu promoter was used to drive the expression of four different selectable marker genes (neomycin phosphotransferase, hygromycin phosphotransferase, phosphinothryic acid N-acetyltransferase and a mutant acetolactate synthase conferring herbicide resistance) that were transformed into both wheat and rice. Wheat callus and transformed rice plants were obtained after selection of transformants, demonstrating that the promoter can be used to drive expression of selectable marker genes to obtain transformed cereals.

20 A review of promoter elements used to control foreign gene expression in transgenic cereals has recently been published and is herein incorporated by reference. (McElroy and Brettel, 1994)

25 A number of promoters are currently being used for transformation of dicotyledonous plants. These promoters come from a variety of different sources. One group of commonly used promoters were isolated from *Agrobacterium tumefaciens*, where they function to drive the expression of opine synthase genes carried on the T-DNA segment that is integrated into the plant genome during infection. These promoters include the octopine synthase (ocs) promoter (L. Comai et al., 1985; C. Waldron et al., 1985), the mannopine synthase (mas) promoter (L. Comai et al., 30 1985; K.E. McBride and K.R. Summerfelt, 1990) and the nopaline synthase (nos) promoter (M.W. Bevan et al., 35

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1983; L. Herrera-Estrella et al., 1983, R.T. Fraley et al., 1983, M. De Block et al., 1984, R. Hain et al., 1985). These promoters are active in a wide variety of plant tissue.

5               Several viral promoters are also used to drive heterologous gene expression in dicots (J.C. Kridl and R.M. Goodman, 1986). The Cauliflower Mosaic Virus 35S promoter is one of the promoters used most often for dicot transformation because it confers high  
10               levels of gene expression in almost all tissues (J. Odell et al., 1985; D.W. Ow et al., 1986; D.M. Shah et al., 1986). Modifications of this promoter are also used, including a configuration with two tandem 35S  
15               promoters (R. Kay et al., 1987) and the mas-35S promoter (L. Comai et al., 1990), which consists of the mannopine synthase promoter in tandem with the 35S promoter. Both of these promoters drive even higher  
20               levels of gene expression than a single copy of the 35S promoter. Other viral promoters that have been used include the Cauliflower Mosaic Virus 19S promoter (J. Paszkowski et al., 1984; E. Balazs et al.) and the 34S promoter from the figwort mosaic virus (M. Sanger et al., 1990).

25               Studies of AHAS expression in a number of plants indicates that AHAS is expressed in all plant tissues. Gail Schmitt and Bijay K. Singh (1990) noted that enzyme assays performed on various tissues of  
30               lima bean demonstrated that AHAS activity was present in all tissues tested, including leaves, stems, roots, flowers, pods and meristems. AHAS activity was found to be fairly constant in the stems, but declined in  
leaves, roots and meristems with increasing age.

35               Sharon J. Kheeler et al., (1993) noted that tobacco contains two genes encoding acetohydroxyacid synthase, SurA and SurB. Both genes appear to be expressed in all tissue types, with about a four-fold



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variation in the level of expression in different tissues. Developing organs appear to have the highest levels of expression. In situ hybridization studies demonstrated that the highest levels of expression were consistently observed in metabolically active or rapidly dividing cells. SurB was expressed at higher levels than SurA in all tissues examined.

Therese Ouellet et al., (1992) noted that Brassica species contain multigene families encoding acetohydroxyacid synthase. Four of the five AHAS genes have been identified in *Brassica napus*. RNase protection assays using gene-specific probes were performed to determine the patterns of expression of the different members of the gene family in various Brassica species. Two of the genes, AHAS1 and AHAS3, were found to be expressed constitutively in all tissues examined. AHAS2 transcripts were detected only in the reproductive organs and extra-embryonic tissue of seeds. Transcripts encoded by the fourth gene, AHAS4, were not detected and it is therefore believed that this represents a pseudogene.

Dale L. Shaner and N. Moorthy Mallipudi (1991) noted that comparison of AHAS activity in young corn leaves and BMS cells grown in suspension culture showed that the activity of the BMS cells per gram fresh weight was approximately 5.8-fold higher than in the leaf samples. Since the BMS cells are actively dividing, this result is consistent with results of previous studies with tobacco and lima bean which demonstrated that younger, actively dividing tissues have more AHAS activity than older tissues.

#### SUMMARY OF THE INVENTION

AHAS promoters from maize are used to express introduced genes at high levels and in various plant tissues. Promoters from the *als1* and *als2* genes

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of maize are cloned and sequenced (figure 1, figure 2, figure 3), and the promoter regions from these genes are then introduced into a plasmid 5' to the reporter gene beta-glucuronidase (GUS). Both promoter fragments are from the XI12 maize line. The als1 promoter fragment is approximately 1400 base pairs long, whereas the als2 promoter fragment contains 819 base pairs. To determine the activity of the AHAS promoters, the chimeric plasmids are then transferred into maize Black Mexican Sweet protoplasts and rice protoplasts for analysis of transient expression levels. For comparison, protoplasts are also transformed with a plasmid with the CaMV 35S promoter driving the GUS reporter gene. The expression of the chimeric plasmids in the maize cells is determined by analyzing the GUS enzyme activity. The activity of the corn als2 promoter was equal to or higher than the activity of the CaMV 35S promoter in both cell types (Figure 4). Figure 5 shows the results of beta-glucuronidase assays performed on maize BMS cell lines stably transformed with either pCD221B (als2-GUS-ocs terminator plasmid) or pAC400 (CaMV 35S-GUS-ocs terminator plasmid). Analysis of AHAS mRNA distribution by *in situ* hybridization of radiolabeled RNA probes to plant tissue shows that the maize AHAS promoters are active in most plant parts (figure 6, figure 7, figure 8).

The activity of the *Arabidopsis* AHAS promoter is analyzed in *Arabidopsis* plants that are stably transformed using *Agrobacterium tumefaciens*. The upstream promoter region of the *Arabidopsis* AHAS gene is fused to the GUS reporter gene, inserted into the binary vector pBIN19 and used to transform *Arabidopsis*. Analysis of transformed plants shows GUS expression, (indicative of the promoter activity) in most plant parts.

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**BRIEF DESCRIPTION OF THE DRAWING**

Figur 1 discloses th AHAS promoter sequence ALS1 from maize XA17.

5 Figure 2 discloses the AHAS promoter sequence ALS2 from maize XA17.

Figure 3 discloses the AHAS promoter sequence ALS2 from maize XI12.

10 Figure 4 is a bar graph presenting beta-glucuronidase activity from transient assays of protoplasts of Black Mexican Sweet maize cells or rice suspension cells after transformation with pCD221B (als2-promoter-GUS-ocs terminator plasmid) or PAC400 (CaMV 35S-GUS-ocs terminator plasmid). The GUS activity is calculated as pmol/min/mg protein.  
15 Results are from three independent experiments.

Figure 5 is a bar graph representing the beta-glucuronidase activity in stably transformed Black Mexican Sweet cell lines after transformation with either pCD221B (als2 promoter-GUS-ocs terminator) or PAC400 (CaMV 35S-GUS-OCS terminator). CK  
20 designates untransformed control tissue. The GUS activity is calculated as pmol/min/mg protein.

Figure 6 - In situ hybridization studies of leaf whorl from 2 week-old corn seedling using  
25 radiolabeled RNA probes. Tissue section were prepared and hybridized to RNA probes encoding either the AHAS sense strand (AHAS-) or the AHAS antisense strand (AHAS+). For a comparison, the SSU (RUBISCO small subunit) sense strand (SSU-) or SSU antisense strand (SSU+) probes were also used. In each case only the  
30 antisense strand (+) is expected to hybridize to the mRNA present in the tissue. a) SSU+probe; b) SSU-probe; c) AHAS+probe; d) AHAS-probe.

35 Figure 7 - In situ hybridization studies of corn kernel 12 days after pollination prepared as described for Figure 6, a) embryo and suspensor,

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AHAS+ probe; b) embryo and suspensor, AHAS- probe; c) pericarp, aleurone and endosperm, AHAS- probe; d) pericarp, aleurone and endosperm AHAS+ probe.

5      Figure 8 - In *situ* hybridization studies of apical meristem of young corn plant prepared as described in Figure 6. a) and b) AHAS+ probe; c) and d) AHAS- probe.

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DETAILED DESCRIPTION OF THE INVENTION

5 Scientists are exploring ways to use genetic modification to confer desirable characteristics on plants. Genetic engineering techniques used to improve characteristics such as taste, texture, size, pest resistance and herbicide-resistance, color, acidity or sweetness of food crops are being explored as a much faster strategy than traditional methods of cross-breeding.

10 The present invention is directed to AHAS promoters from maize and *Arabidopsis* and vectors and plant cells comprising these promoters. (For purposes of this application a promoter is defined as a nucleotide sequence found upstream of a gene that acts as a signal for the binding of the RNA polymerase.) The *als1* and *als2* genes of maize are cloned and sequenced and the promoter regions from these genes are then introduced into a plasmid 5' to the GUS reporter gene. For purposes of this application a reporter gene is defined as a nucleotide sequence which is fused downstream of the promoter of interest, so that transcripts initiating at the promoter proceed through the reporter gene. Reporter genes encode some easily measurable enzyme activity; for example, in the present invention the expression of chimeric plasmids in maize cells is determined by analyzing the beta-glucuronidase (GUS) enzyme activity.

25 One skilled in the art should appreciate that AHAS promoters are highly active in many different plant tissues, and can be used to express novel genes in a variety of plants. Novel genes include but are not limited to genes for herbicide resistance, detoxification and resistance towards plant pathogens.

30 The claimed promoters can be used for

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heterologous gene expression in monocotyledons, including but not limited to maize, rice, wheat, barley, sorghum, oats, rye and millet. One skilled in the art should also appreciate that the claimed promoters will also drive expression in dicotyledons, although expression of monocot promoters in dicots is expected to be at lower levels than in monocots.

One skilled in the art should appreciate that the maize *als2* promoter can be used to drive gene expression in other monocot species. For example, the rice *Act1* promoter drives gene expression in maize protoplasts, the maize *Emu* promoter has been used to select transgenic wheat, barley and rice, and the *Arabidopsis* *AHAS* promoter, a dicot promoter, has been used to drive *AHAS* gene expression in transgenic tobacco and transgenic potato. The claimed maize *als2* performs best in maize, but also drives gene expression in another monocot. Based on studies done in maize and other species, this promoter should drive constitutive expression of a gene throughout a plant. The highest levels of expression is seen in either actively dividing or metabolically active tissue.

A variety of different techniques well known by those skilled in the art including, but not limited to, microprojectile bombardment, PEG-mediated transformation, electroporation, and silicon fibers have been used to transform monocot crops. All of these techniques involve the use of DNA vectors for delivery of the nucleotide sequences to be transformed. Vectors suitable for use in the present invention include, but are not limited to, vectors carrying marker genes used for the selection of transgenic material, including genes conferring resistance to hygromycin, kanamycin, bialaphos and the imidazolinone and sulfonylurea herbicides.

The following Example serves only to

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illustrate the invention and is not to be construed as in any way limiting on the invention.

#### EXAMPLE

5

The efficiency of the *als1* and *als2* promoters is assessed by measuring GUS activity in maize and rice protoplasts or maize cell lines carrying constructs in which these promoter sequences are fused to the GUS gene. The preparation of the vectors and transformation protocol is described below.

10

Constructs containing the Chimeric CaMV 35S-GUS, *als1*-GUS and *als2*-GUS Genes

15

Plasmid pAC400 is a pUC19-based plasmid containing a fusion of the 418 base pair (pb) EcoRI-XbaI fragment of the CaMV 35S promoter cloned upstream of a 1.7 kb XbaI-PstI fragment containing the GUS gene and a 700 bp PstI-BamHI fragment containing the octopine synthase terminator. The *als1* and *als2* genes are obtained by screening a genomic library prepared from maize line XI12. An EcoRI-NcoI fragment containing 1.4 kilobase (kb) of sequence upstream from the ATG initiation codon of the *als1* gene is subcloned into pAC400 in place of the CaMV 35S promoter to create pCD223B. An 819 bp PstI-NcoI fragment upstream from the ATG of *als2* was subcloned in front of the same GUS-ocs terminator fusion in pBluescript® KS- (Stratagene) to create pCD221B. The sequence of the *als2* promoter from maize XI12 is presented in Figure 3.

20

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Transformation and Assay of Protoplasts of Rice and Maize

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Protoplasts are isolated from rice (Nortai) or maize (Black Mexican Sweet; BMS) suspension cells and transformed with pAC221B or pAC400 according to the PEG-mediated transformation protocols of L.A. Lyznik et al. (1989) and J.Y. Peng et al. (1990). For transient assays, transformed rice protoplasts are plated on Millipore filters placed on top of medium containing feeder cells and transformed BMS protoplasts are cultured in 3 ml of liquid medium. Two days after transformation, protoplast cultures are collected and extracted with GUS extraction buffer. GUS activity is measured fluorimetrically according to the protocol of R.A. Jefferson (1987).

To recover stable transformants from maize BMS culture, protoplasts are co-transformed with pPF19K (containing the selectable marker gene encoding neomycin phosphotransferase) and pCD221B or pAC400. After transformation, protoplasts are cultured on Millipore filters placed on top of medium containing feeder cells. One week later, the protoplasts cultures are transferred to medium containing feeder cells and 100 mg/l kanamycin. Protoplasts cultures are transferred to fresh MS medium with 100 mg/l kanamycin until resistant calluses became visible. Individual kanamycin resistant callus is picked and grown for two to three more weeks in the presence of kanamycin. Kanamycin-resistant callus is stained with X-Gluc or assayed with MUG according to the protocol of R.A. Jefferson (1987) to screen for GUS-positive callus. Callus identified as expressing GUS is ground in extraction buffer and assayed; GUS activity is measured fluorimetrically.

Data from these experiments is presented in Figures 4 and 5.

A mutant maize AHAS gene conferring resistance to imidazolinone herbicides and driven by



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the maize *als2* promoter is used as a selectable marker to obtain transgenic callus after transformation of both BMS and A188 x B73 cells and rice cells. This result supports the idea that the *als2* promoter drives sufficiently high levels of expression to drive marker gene expression.

#### *In situ* Hybridization

Slides are prepared essentially described by J. A. Langdale et al (1988). Tissue is fixed in 4% formaldehyde, dehydrated in ethanol, cleared with xylene and embedded in paraffin. Tissue is sliced into 8-10  $\mu$ m sections and placed onto slides coated with poly-L-lysine. Paraffin is removed with xylene and tissue is rehydrated by washing in ethanol and rinsing in water. RNA probes are prepared from both strands (+ and -) of a 172 nucleotide (nt) fragment from the *als2* encoding region and a 392 nt fragment from the SSU coding region using the Ambion Maxiscript<sup>®</sup> kit. Slides are hybridized according to the protocol of Meyerowitz et al (1988) at 50°C overnight in a 1:4 dilution of SPB [100  $\mu$ l of 10 X salts (3M NaCl, 10mM Tris p.H. 6.8, 50 mM DTA); 400  $\mu$ l formamide; 200  $\mu$ l 50% dextran sulphate; 40  $\mu$ l 10 mg/ml tRNA; 10  $\mu$ l 1 MDTT; 50  $\mu$ l 10 mg/ml poly A] with probe denatured in 50% formaldehyde and 10mM DTT at 80°C for 30 seconds. Slides are washed 2X 15 minutes in Wash Buffer (1X salts, 50% formamide, 10 mM Tris pH 8, 1mM EDTA and 10 mM DTT) at 50°C, treated with RNase A (20  $\mu$ g/ml in NTE\_ for 30 minutes at 37°C, washed 5X in NTE buffer at 37°C for a total of 1 hour, washed 2 X 30 minutes in Wash Buffer at 50°C, dehydrated in ethanol and air dried. Slides are autoradiographed by dipping into emulsion prewarmed to 37°C in darkroom, drying for 30 minutes to 1 hour and storing in the dark at 4°C until developing. Slides are developed for 2 minutes, rinsed in water, fixed for at least 5 minutes

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and rinsed again in water. Tissue is stained with Alcian Blue and then stained in ethanol and xylene. After mounting with Permount, tissue is observed using a dark field microscope.

5                   The data from in situ hybridization experiments in Figure 6-8 shows that the gene is also expressed throughout the maize embryo.

It is possible to evaluate the expression of the AHAS promoter by:

10                   1. constructing an *als2*-GUS fusion gene and determining the pattern of GUS activity within transgenic corn or rice plants. Previous studies using a maize promoter have demonstrated the feasibility of evaluating maize promoter expression in  
15                   rice (Junko Kyojuka, et al., 1991). After selection of transgenic plants, histochemical analyses are performed on plant tissues at various stages of development to determine both tissue- and cell type-specificity. This technique is commonly used to  
20                   evaluate promoter activity in both monocots and dicots.

                  2. transient expression assays are performed on protoplasts prepared from different plant species following transformation of *als2*-GUS  
25                   constructs. This approach is used to evaluate the ability of different promoters to function in heterologous species. Protoplasts are transformed with the construct of interest and incubated to allow the introduced gene to be expressed and the protein to  
30                   accumulate. Following the incubation, the cells are assayed for the presence of the protein encoded by the transgene to determine the efficiency of the promoter driving transgene expression.

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REFERENCES

1. David McElroy et al., (1990) Isolation of an efficient actin promoter for use in rice transformation. The Plant Cell 2: 163-171.
2. David McElroy et al., (1991) Construction of expression vectors based on the rice actin 1 (Act1) 5' region for use in monocot transformation. Mol. Gen Genet. 231: 150-160.
3. Wanggen Zhang et al., (1991) Analysis of the Act1 5' region activity in transgenic rice plants. The plant Cell 3: 1155-1165.
4. Jun Cao et al., (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Reports 11: 586-591.
5. Alan H. Christensen et al., (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Molecular Biology 18: 675-689.
6. Seiichi Toki et al., (1992) Expression of a maize ubiquitin gene promoter-bar chimeric gene in transgenic rice plants. Plant Physiol. 100: 1503-1507.

- 18 -

7. J. Troy et al., (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). *Plant Physiol.* 102: 1077-1084.
8. Yuechun Wan and Peggy G. Lemaux, (1994) Generation of a large number of independently transformed fertile barley plants. *Plant Physiol.* 104: 37-48.
9. Junko Kyoizuka et al., (1991) Anaerobic induction and tissue-specific expression of maize *Adh1* promoter in transgenic rice and their progeny. *Mol. Gen. Genet.* 228: 40-48.
10. D. I. Last et al., (1991) pEmu: an improved promoter for gene expression in cereal cells. *Theor. Appl. Genet.* 81: 581-588.
11. Robert Bower and Robert G. Birch (1992) Transgenic sugarcane plants via microprojectile bombardment. *The Plant Journal* 2 (3): 409-416.
12. D. A. Chamberlain et al., (1994) The use of the Emu promoter with antibiotic and herbicide resistance genes for the selection of transgenic wheat callus and rice plants. *Aust. J. Plant. Physiol.*, 21: 95-112.
13. L. Comai et al. (1985) Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. *Nature* 317: 741-744.
14. C. Waldron et al. (1985) Resistanc to

- 19 -

hygromycin B: A new marker for plant transformation studies. Plant Mol. Biol. 5: 103-108.

15. Kevin E. McBride and Kristin R. Summerfelt (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol. Biol. 14: 269-276.
16. Michael Bevan et al. (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304: 184-187.
17. Luis Herrera-Estrella et al. (1983) Expression of chimeric genes transferred into plant cells using Ti-plasmid-derived vector. Nature 304: 209-213.
18. Robert T. Fraley et al. (1983) Expression of bacterial genes in plant cells. P.N.A.S. 80: 4803-4807.
19. Marc De Block et al. (1984) Expression of foreign genes in regenerated plants and in their progeny. EMBO J.3: 1681-1689.
20. R. Hain et al. (1985) Uptake, integration, expression and genetic transmission of a selectable chimeric gene by plant protoplasts. Mol. Gen. Genet. 199: 161-168.
21. J.C. Kridl and Robert M. Goodman (1986) Transcriptional regulatory sequences from plant viruses. Bio Essays 4:4-8.

- 20 -

22. Joan T. Odell et al. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810-812.
23. David W. Ow et al (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234: 856-959.
24. D.M. Shah et al. (1986) Engineering herbicide tolerance in transgenic plants. Science 233: 478-481.
25. Robert Kay et al. (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236: 1299-1302.
26. L. Comai et al. (1990) Novel and useful properties of a chimeric plant promoter combining CaMV 35S and MAS elements. Plant Mol. biol. 15: 373-381.
27. J. Pazskowski et al. (1984) Direct gene transfer to plants. EMBOJ. 3: 2717-2722.
28. Ervin Balazs et al. (1985) Chimeric vector construction for higher-plant transformation. Gene 40: 343-348.
29. Margaret Sanger et al. (1990) Characteristics of a strong promoter from figwort mosaic virus: Comparison with the analogous 35S promoter from cauliflower mosaic virus and the regulated mannopine

- 21 -

- synthase promoter. Plant Mol. Biol. 14: 433-443.
30. Gail Schmidt and Bijay K. Singh (1990) Tissue distribution of acetohydroxyacid synthase activity at various developmental stages of lima bean. Pesticide Sci. 30 (4): 418-419.
31. Sharon J. Kheeler et al., (1993) Regulation of tobacco acetolactate synthase gene expression. Plant Physiol. 102:1009-1018.
32. Therese Ouellet et al., (1992) Members of the acetohydroxyacid synthase multigene family of *Brassica napus* have divergent patterns of expression. The Plant Journal 2: 321-330.
33. Dale L. Shaner and N. Moorthy Mallipudi (1991) Imidazolinone-Acetohydroxyacid synthase interactions. In The Imidazolinone Herbicides ed. Dale L. Shaner and Susan L. O'Connor CRC Press (Boca Raton, FL.)
34. R. A. Jefferson (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rept 5: 387-405.
35. L.A. Lyznik et al. (1989). Stable transformation of maize protoplasts with *gusA* and *neo* genes. Plant Mol Biol 13: 151-161.
36. J.Y. Peng et al. (1990). Co-transformation

- 22 -

of *indica* rice protoplasts with *neo* and *gusA* genes. Plant Cell Rept 9: 168-172.

37. J. A. Langdale et al., (1988), Cellular pattern of photosynthetic gene expression in developing maize leaves. Gene Dev. 2: 106-115.

38. E. Meyerowitz et al., (1988), In situ hybridization to RNA in plant tissue. Plant Mol. Bio. Rept., 5: 242-250.



- 23 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Dietrich, Gabriele
- (ii) TITLE OF INVENTION: Non-coding DNA 5' to the structural gene (promoter) for acetoxyacid synthase (AHAS) is useful for the expression of introduced genes in plants.
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: American Cyanamid Company
  - (B) STREET: One Cyanamid Plaza
  - (C) CITY: Wayne
  - (D) STATE: New Jersey
  - (E) COUNTRY: United States
  - (F) ZIP: 07470-8426
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Harrington, James J.
  - (B) REGISTRATION NUMBER: P38,711
  - (C) REFERENCE/DOCKET NUMBER: 32,348
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 201-831-3246
  - (B) TELEFAX: 201-831-3305

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 413 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTAGAGAAA CTAACTACT AATAAAATT ATTTTAGCA TATTTAGTA CTGTNGTTA  
60

TATTTNNAAG TGATAAAGTT TAACTAAAAG TGCACCGCTA AACCACCGTA AATCCAAAGA  
120

- 24 -

GACCGTAAAT CTCTTCCACG CACTCTGTCTG TGTACCAACG TGCTGTGGAA ACGCTCACGT  
180

ACCTTTGTGT ATTATGTACG GATTCTGGGCA ACGGACATT CGACGTCGGT TTGCCAGTCC  
240

NATTCCCATC TGAACCACAC ATCTCTGAAC AAAAGTAGGG GAGGCGCCCC CGTAGCCCCC  
300

TTTCCACAA TCCCACTCCG TGCCAGGTGC CACCCTCCCC AAGCCCTCGC GCCGCTCCGA  
360

GACAGCCGCC CGCAACCATG GCCACCGCCG CCACCGCGGC CGCCGCGCTC ACC  
413

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 509 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCGGATTTC CTGTTGCGGA TTGCGGGTGG CAGCCTGGCA GGTGGGTGCG ACCCCGTTTG  
60

GATTCCCTTG TCTGGGCCCC TTGTGTCAGT ACCGTCTGTA CTCCGATGAC ATGCACCGTC  
120

GTCCACAGTC AAGTCCAAAA TCTCCCCTCT TTTTTTTAAC GGAACAGTTC AAAACCTCCT  
180

TGACGCACGC TGTCGTGTAC CAGCACTCGG TGGACACCAC GTTTGTAATC CAGGCCGACA  
240

CGTCGGTCCC ACGTCGACAG GCCCCACCGT CCGGTCTGTA GCGTGTACGT ATTCGGGCGA  
300

CGGACGTGTC GTCGTCGTCT TGCGAGTCCC ATTCCCATCA CCATCTGAGC CACACATCCT  
360

CTGAACAAAA GCAGGGAGGC CTCCACGCAC ATCCCCCTTT CTCCACTCCG GTCCGTGGCA  
420

CCCACCCCAA ACCCTCGCGC CGCCTCCGAG ACAGCCGCCG CAACCATGGC CACCGCCGCC  
480

GCCGCGTCTA CCGCGCTCAC TGGCGCCAC  
509

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 829 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- 25 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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120

AAAAACAGTT GAACAAGATT GACTGTTCTT CCGGGAGGGT TTGGAACATC GTTACAGATG  
180

TGAGCGAAAG GTGAGGAAAC AGAGCGGAGG GCTTGGAGGT GACCTCGGTA GTCAGACGCC  
240

GGAGTTGAGC TTGATGACGA CACCGTACTG GCGTACCAGG CCTAGTAGTG AACACCGGGC  
300

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360

TGCGGGTGGC AGCCTGGCAG GTGGGTGCGA CCCGTTTGA CTCCCTGATC TGGGCCCTTT  
420

GTGTCAGTAC CGTCTGTACT CCGATGACAT GCACANCGTC GTCCACAGTC AAGTCCACAA  
480

TCTCCCCTCT TTTTAAACG GAATAGTTNC AAAATCTCCT TGACGCACGC TATCGTGTAC  
540

CAGCGCTCAC TGGACACCAC GTTTGTAATC CACGCCGACA CGTCGNTCCC ACGTCGACAG  
600

GCCCCACCGT CCGGTCTGTA GCGTGTACGT ATTCTGGCAA CGGACGTGTC GTCGTGCTCT  
660

TGCNNNNGTC CCANNNCCCA TCACCATCTG AGCCATCACA TCTCATGCGT GAANAAAAGC  
720

AGGGAAGGCC TCTACGCACA TCCCCCTTC TNNCTNNNT CCGTGTCCGT GGCACCCAGG  
780

GGAAACCCTC GCGCCGCCTC CGAGACAGCC GCCGCAACCA TGGCCACCG  
829

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CLAIMS

1. An isolated nucleotide sequence comprising an AHAS promoter for expression of genes in plants.
2. The nucleotide sequence of Claim 1 comprising the sequence of Figure 1, SEQ. ID. No. 1.
3. The nucleotide sequence of Claim 1 comprising the sequence of Figure 2, SEQ. ID. No. 2.
4. The nucleotide sequence of Claim 1 comprising the sequence of Figure 3, SEQ. ID. No. 3.
5. The nucleotide sequence of Claim 1, 2, 3 or 4 in which the plant is a monocot.
6. The sequence of Claim 5, in which the monocot is corn.
7. A transformation vector comprising nucleotide sequence of Claim 1, 2, 3, 4 or 5.
8. A plant cell comprising the vector of Claim 7.
9. A mature plant comprising the nucleotide sequence of Claim 1, 2, 3, 4 or 5.
10. A method of expressing a heterologous gene in a plant at high levels and in different tissues of said plant comprising expressing

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a heterologous gene in said plant under the control of a nucleotide sequence of Claim 1, 2 3 or 4.

11. A nucleic acid construct comprising the sequence of Claim 1, 2, 3 or 4 linked to a heterologous gene.
12. A nucleic acid construct of Claim 11 wherein the gene is an AHAS gene.
13. A method of using a nucleic acid construct as a selectable marker, the construct comprising the AHAS promoter sequence of Claim 1, 2, 3 or 4 linked to a mutant gene, the gene conferring resistance to a selectable, transgenic material, the method comprising the steps of:
  - (a) recombinantly transforming a plant material by inserting the nucleic acid construct;
  - (b) placing the plant material on a growth medium comprising a compound;
  - (c) identifying the plant materials capable of growth in the presence of the compound.
14. The method of Claim 13 wherein the mutant gene is an AHAS gene.
15. The method of Claim 13 wherein the compound is a imidazolinone or sulfonyl urea compound.

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TCTAGAGAACTAACTACTAATAAAAATTATTTTTAGCATATT  
TTAGTACTGTNGTTTATATTTNNAATGATAAAGTTTAACTAAA  
AGTGCACCGCTAAACCACCGTAAATCCAAAGAGACCGTAAATCT  
CTTCCACGCACTCTGTCGTGTACCAACGTGCTGTGGAAACGCTC  
ACGTACCTTTGTGTATTATGTACGGATTCGGGCAACGGACATTT  
CGACGTCGGTTTGCCAGTCCNATTCCCATCTGAACCACACATCT  
CTGAACAAAAGTAGGGGAGGCGCCCGCGTAGCCCCCTTTCCAC  
AATCCCCTCCGTGCCAGGTGCCACCCTCCCCAAGCCCTCGCGC  
CGCTCCGAGACAGCCGCGCAACCATGGCCACCGCCGCCACCG  
CGGCCGCCGCGCTCACC

FIG. 1

CCGGATTTCCCTGTTGCGGATTGCGGGTGGCAGCCTGGCAGGTG  
GGTGCGACCCGTTTGGATTCCCTTGTCTGGGCCCTTGTGTCA  
GTACCGTCTGTACTCCGATGACATGCACCGTCGTCCACAGTCAA  
GTCCAAAATCTCCCCTCTTTTTTTTAAACGGAACAGTTCAAAACC  
TCCTTGACGCACGCTGTGTCGTGTACCAGCACTCGGTGGACACCAC  
GTTTGTAATCCAGGCCGACACGTCCGTCCCACGTGACAGGCC  
CACCGTCCGGTCTGTAGCGTGTACGTATTCGGGCGACGGACGTG  
TCGTGTCGTCTTGCGAGTCCATTCCCATCACCATCTGAGCCA  
CACATCCTCTGAACAAAAGCAGGGAGGCCTCCACGCACATCCCC  
CTTTCTCCACTCCGGTCCGTGGCACCCACCCAAACCCTCGCGC  
CGCCTCCGAGACAGCCGCGCAACCATGGCCACCGCCGCCGCCG  
CGTCTACCGCGCTCACTGGCGCCAC

FIG. 2

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CTGCAGGTCA ACGGATCACC TATCAACATC CCAGCTAAAA ACAGTAAAAA  
GGGGGAAAAC GTGGGTGAGT TGAGTCTGTC TTGTGGAAAA AACGTTTTAG  
TTTCTCCTGG AATTAACAAT AAAAACAGTT GAACAAGATT GACTGTTCCCT  
CCGGGAGGGT TTGGAACATC GTTACAGATG TGAGCGAAAG GTGAGGAAAC  
AGAGCGGAGG GCTTGGAGGT GACCTCGGTA GTCGACGCCG GAGTTGAGCT  
TGATGACGAC ACCGTA CTGGTACGAGG CTAAGTAGTA ACACCGGGCC  
TGAAGCTGTC GCCGCCGCTG CTCATCTTGT GGGCTGTGCC CCGTGTCCCT  
GTTGCGGATT GCGGGTGGCA GCCTGGCAGG TGGGTGCGAC CCGTTTGGAC  
TCCCTGATCT GGGCCCTTTG TGTCAGTACC GTCTGTACTC CGATGACATG  
CACACCGTCG TCCACAGTCA AGTCCACAAT CTCCCCTCTT TTTTAAACGG  
AATAGTTCAA AATCTCCTTG ACGCACGCTA TCGTGTACCA GCGCTCACTG  
GACACCACGT TTGTAATCCA CGCCGACACG TCGGTCCCAC GTCGACAGGC  
CCCACCGTCC GGTCTGTAGC GTGTACGTAT TCGGGCGACG GACGTGTCGT  
CGTCGTCTTG CGAGTCCCAT TCCCATCACC ATCTGAGCCA CACATCCTCT  
GAACAAAAGC AGGGAGGCCT CTACGCACAT CCCCCTTTCT CCCACTCCGT  
GTCCGTGGCA CCCACCCCAA ACCCTCGCGC CGCCTCCGAG ACAGCCGCCG  
CAACCATGGC CACCG

FIG.3

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	RICE			BMS		
	EXPT. I	EXPT. II	EXPT. III	EXPT. I	EXPT. II	EXPT. III
ALS2-GUS						
	3040	8337	1129	3174		2275
	2413	5110	1033	1269		6117
	2847	8024	1187	4145	7295	3234
AVERAGE	2767	7157	1999	2863	9419	2060
STD. DEV.	321	1780	1337	1463	8357	4018
			446			3369
						3149
						997
35S-GUS						
	970		1882			334
	1175		1097			624
	1732	2430	1235	348		1032
	1292	3435	731	540	1021	341
	394	2933	1236	292	1228	384
AVERAGE			480	393	1125	359
STD. DEV.				130		529
						336
RATIO OF ALS/35S						

FIG. 4A



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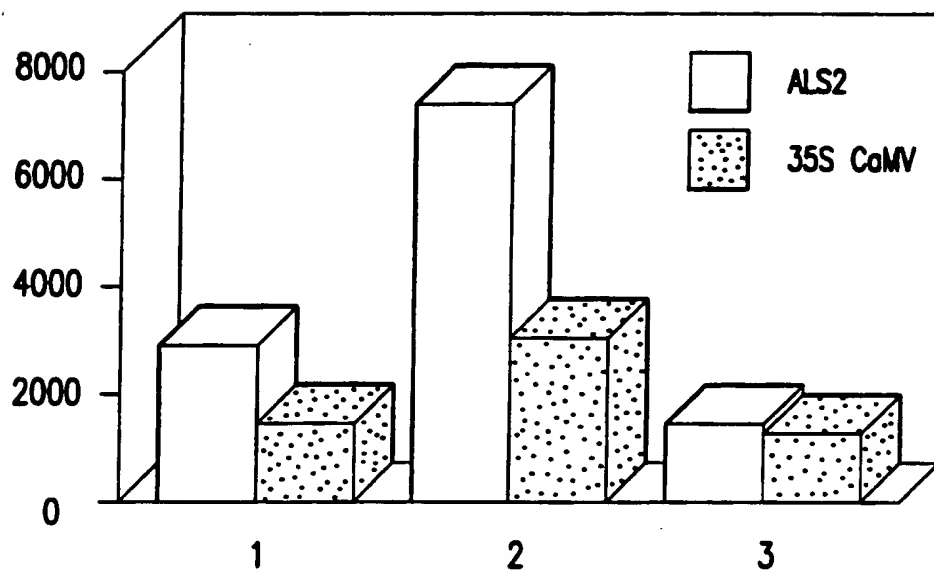


FIG. 4B

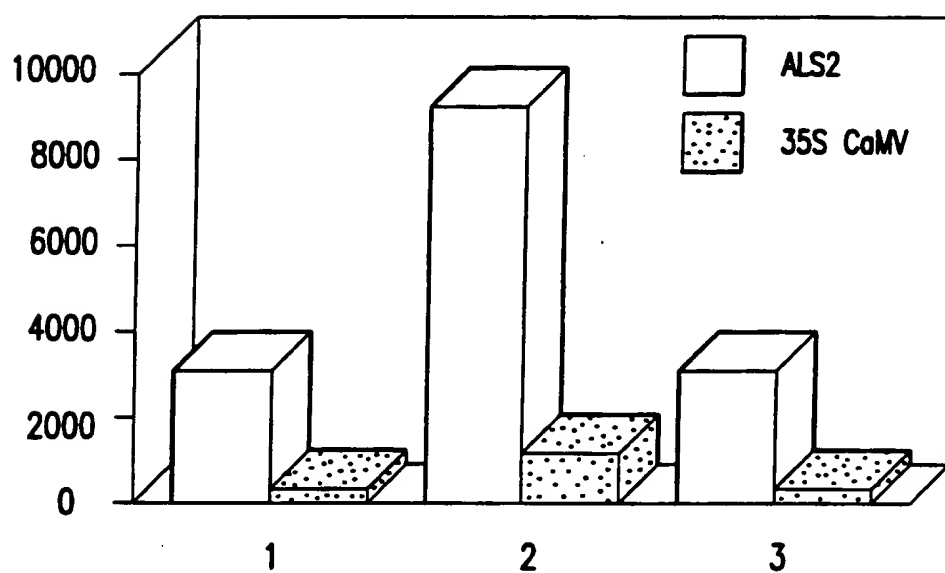


FIG. 4C

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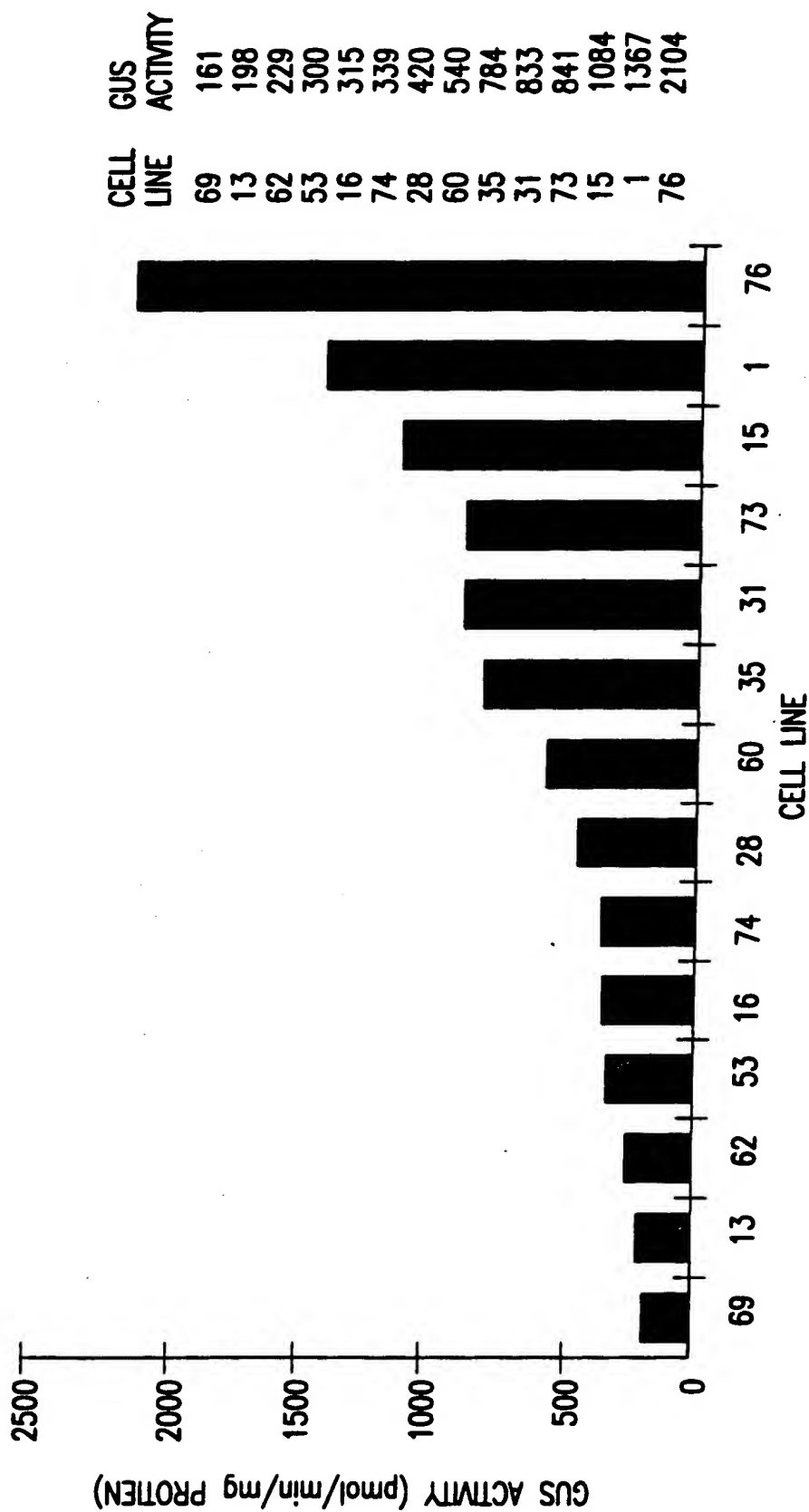


FIG.5

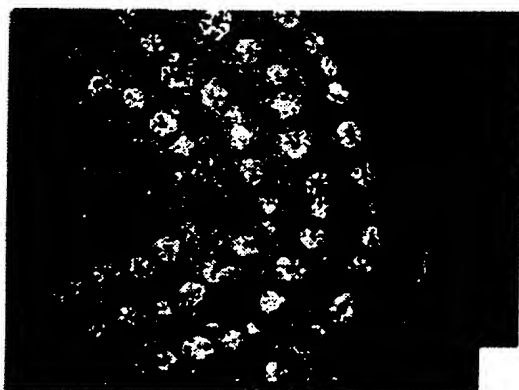


FIG. 6A



FIG. 6B



FIG. 6C

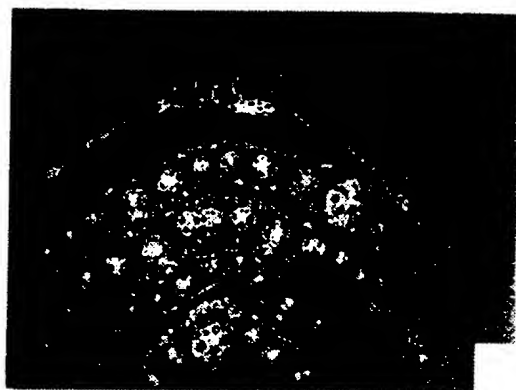


FIG. 6D



FIG. 7A



FIG. 7B



FIG. 7C



FIG. 7D



FIG. 8A



FIG. 8B

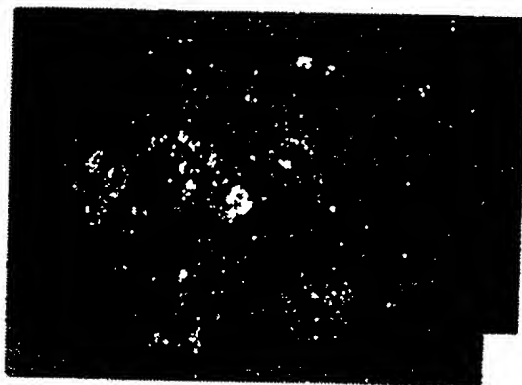


FIG. 8C



FIG. 8D

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 95/11199

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N15/60 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT MOLECULAR BIOLOGY, vol. 18, 1992 pages 1185-1187, FANG, L.Y., ET AL. 'Sequence of two acetoxyacid synthase genes from Zea mays' see the whole document ---	1-7,9
X	EP,A,0 360 750 (CIBA GEIGY AG) 28 March 1990 see figures 9,10 --- -/--	1-7,9

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*A\* document member of the same patent family

Date of the actual completion of the international search

9 January 1996

Date of mailing of the international search report

02.02.96

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Maddox, A

## INTERNATIONAL SEARCH REPORT

Int. Application No.

PCT/US 95/11199

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOLOGY, vol. 102, 1993 pages 1009-1018, KEELER, S.J., ET AL. 'Regulation of tobacco acetolactate synthase gene expression' cited in the application see page 1016, right column ---	1
X	PLANT PHYSIOLOGY, vol. 91, 1989 pages 574-580, GABARD, J.M., ET AL. 'Cross-resistance to short residual sulfonylurea herbicides in transgenic tobacco plants' see the whole document ---	1,7-15
Y	---	1-15
X	EP,A,0 525 384 (AMERICAN CYANAMID CO) 3 February 1993 see seq. I.D. no. 1 see claims 1-14 ---	9
Y	---	4-15
X	EP,A,0 461 355 (AMERICAN CYANAMID CO) 18 December 1991 see the whole document ---	9
Y	---	1-3,5-15
X	THE PLANT CELL, vol. 2, May 1990 pages 415-425, LEE, K.Y., ET AL. 'Homologous recombination in plant cells after Agrobacterium-mediated transformation' see table 1 ---	10-12
X	US,A,5 013 659 (BEDBROOK JOHN R ET AL) 7 May 1991 see examples III, IV, VII ---	10-12,15
X	BIOTECHNOLOGY, vol. 10, no. 3, March 1992 pages 309-314, D'HALLUIN, K., ET AL. 'Transformation of sugarbeet (Beta vulgaris L.) and evaluation of herbicide resistance in transgenic plants' see page 313 left col. "strains and plasmid vectors" ---	10-12
X	EP,A,0 492 113 (AMERICAN CYANAMID CO) 1 July 1992 see the whole document ---	13-15
	---	

-/--

# INTERNATIONAL SEARCH REPORT

Int. Application No.

PCT/US 95/11199

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOLOGY, vol. 97, 1991 pages 1044-1050, SATHASIVAN, K., ET AL. 'Molecular basis of imidazolinone herbicide resistance in Arabidopsis thaliana var. Columbia' see the whole document ---	15
A	WO,A,92 08794 (ICI PLC) 29 May 1992 see the whole document -----	1-15



**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

Int. Application No.  
**PCT/US 95/11199**

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0360750	28-03-90	AU-B- 4171489 JP-A- 2186925	03-05-90 23-07-90
EP-A-0525384	03-02-93	AU-B- 2069492 BG-A- 96699 CA-A- 2074854 CZ-A- 9202385 HU-A- 65483 JP-A- 5227964	04-02-93 30-06-94 01-02-93 16-03-94 28-06-94 07-09-93
EP-A-0461355	18-12-91	AU-B- 641496 AU-B- 7605891 US-A- 5290753	23-09-93 14-11-91 01-03-94
US-A-5013659	07-05-91	US-A- 5141870	25-08-92
EP-A-0492113	01-07-92	AU-B- 9005591 CA-A- 2058349 JP-A- 4311392	02-07-92 28-06-92 04-11-92
WO-A-9208794	29-05-92	AU-B- 663856 AU-B- 8868191 CA-A- 2096348 EP-A- 0557352 JP-T- 6502074	26-10-95 11-06-92 15-05-92 01-09-93 10-03-94